

# The Clastogenic Potential of Triazine Herbicide Combinations Found in Potable Water Supplies

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Pesticide contamination of drinking water supplies has increased over the past decade. A major concern is how exposure to combinations of low levels of pesticides, especially herbicides, could affect public health. Flow cytometric analysis was performed to determine the clastogenic potential of herbicide interaction on Chinese hamster ovary (CHO) cells. The cells were exposed to atrazine, simazine, cyanazine, and all possible combinations of these chemicals for 48 hr. Two concentrations were used for each sample: the U.S. EPA maximum contamination level (MCL) and the highest contamination level found in Illinois water supplies. Nuclei were isolated from the cells and analyzed by flow cytometry. The effects of clastogenicity were measured by the coefficient of variation (CV) of the G1 peak of whole cells and the change in CV of the largest chromosome in the flow karyotype. At both levels tested, atrazine caused chromosomal damage to the CHO cells. Simazine was observed to induce whole-cell clastogenicity but not flow karyotype damage. Cyanazine did not induce any measurable chromosomal damage in either analysis. Each of the herbicides, although all three were triazines, had different effects with respect to chromosome damage as measured by flow cytometry. CHO cells treated with a combination of atrazine and simazine, or atrazine and cyanazine, were observed to have whole-cell and flow karyotype damage. This damage was, however, equal to or less severe than the damage caused by either atrazine or simazine alone. No synergy was observed. When all three herbicides were combined, three of the four possible combinations gave no observable clastogenic response. *Key words:* atrazine, Chinese hamster ovary (CHO) cells, chromosome, combination, cyanazine, simazine, synergy. *Environ Health Perspect* 106:197–201 (1998). [Online 5 March 1998] <http://ehpnet1.niehs.nih.gov/docs/1998/106p197-201taets/abstract.html>

Potable water contamination has become a major concern. A study conducted from July 1992 through June 1993, testing 135 water supplies including 6 groundwater sources, detected 22 pesticides in various mixtures and concentrations in 114 of these samples (1). Atrazine was one of the major pesticides found in water supplies, with 34 samples registering >3 ppb (0.003 µl/ml), the maximum contamination level (MCL) for drinking water determined by the U.S. EPA. Simazine and cyanazine were also detected in 28% and 33% of samples, respectively (1). Most of the pesticides that were detected were below the MCL; however, their presence could still pose a health risk. These pesticides at low contamination levels have not been studied thoroughly to determine the health risks that they may present.

Yoder et al. (2) found that there was an increase of chromosomal aberrations in lymphocyte cultures of farm workers exposed to pesticides, including the herbicide atrazine. Chromosomal aberrations have also been detected in the bone marrow cells of mice exposed to a single dose of atrazine (3). Meisner et al. (4) found that when human lymphocyte cultures were exposed to atrazine at levels as low as 0.1 µg/ml, a significant percentage of cells exhibited chromosome aberrations. Chinese hamster ovary (CHO)

cells exposed to atrazine for 48 hr showed whole-cell clastogenic properties (5). Human lung fibroblasts exposed to simazine exhibited unscheduled DNA synthesis (6), although a similar test done by Waters et al. (7) had negative results. In the sex-linked recessive lethal test, lethal mutations arose in fruitflies, *Drosophila melanogaster*, treated with simazine (8). A recent report has indicated an association between intrauterine growth retardation and triazine contamination (9). Given the potential health risk of herbicide contamination, it is very important to ask how these chemicals might interact with each other. Mixtures of herbicides have been found in water supplies at a given time (1). The effects that mixtures of herbicides could have on human health are undetermined at this time.

Flow cytometry is a sensitive technique for detecting clastogenic properties (both whole cell and individual chromosome damage) of various mutagens (5,10–16). Aten (10) demonstrated that the width of chromosomal peaks in flow karyotypes increases with radiation-induced chromosomal aberrations. In a recent study done on maize, Rayburn et al. (17) used flow cytometry to detect small changes in DNA caused by a treatment with a mutagenic fungicide. Induced mutations have been correlated with an increased coefficient of

variation (CV) of the G1 peak of flow histograms (14,18–20). The CV of the G1 peak can thus be used to determine whether a mutagen has whole-cell clastogenic properties. The CV of the G1 peak increases due to chromosomal breakage, which causes uneven distribution of DNA in the daughter cells. Flow cytometry has been demonstrated to be accurate and sensitive in detecting the clastogenicity of whole cells in CHO subcultures and mouse bone marrow cells (15). Several studies have also demonstrated the utility of flow karyotyping in detecting chromosomal damage. The CV of the largest chromosomal peak has been used to observe clastogenic potential.

Biradar and Rayburn (5,11) used flow cytometric analysis to determine that atrazine causes damage to animal chromosomes. Atrazine is currently under special review by the EPA, and atrazine contaminates surface-water supplies throughout the Midwest. Due to these factors, alternatives to atrazine have been sought by the agricultural community. Use of alternative chemicals has resulted in mixtures of various herbicides in surface-water supplies (1). Although the exact mechanisms of clastogenicity of atrazine have yet to be determined, it is critical to determine if mixtures of these herbicides induce chromosome damage.

The main objective of this project was to study the effects of herbicide interaction on CHO cells at both the EPA MCL and at the highest levels that have been detected in Illinois water supplies. The three herbicides atrazine, simazine, and cyanazine were chosen because they are the most frequently found herbicide contaminants of water samples taken in Illinois (1).

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## Materials and Methods

### Cell Culture

A stable line of CHO cells (subclone P1A6) was maintained in a monoculture in F-10 Ham media with 15% calf serum and 200 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), at 37°C and 5% CO<sub>2</sub> in a humidified chamber. Cultures were maintained by adding 0.5 ml cell suspension to 9.5 ml complete medium in petri plates (100 × 15 mm). To treat the cells, the three herbicides atrazine [6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine; CAS 1912-24-9], cyanazine [2-(4-chloro-6-[ethylamino]-1,3,5-triazin-2-yl)amino-2-methylpropane nitrile; CAS 21725-46-2], and simazine [2-chloro-4,6-bis(ethylamino)-1,3,5-triazine; CAS 122-34-9] were added in appropriate concentrations (all stock solutions and subsequent dilutions were made in distilled water; no additional solvents were added) and combinations to subcultured CHO cells (1 ml of cell suspension to 9 ml complete media). Due to the consistent chromosome damage previously observed in atrazine-treated cells, atrazine was used as the positive control (5,11). The herbicides were mixed at the MCL concentrations: 3 ppb (0.003 µg/ml) for atrazine and cyanazine (no MCL for cyanazine is set, so the same level designated for atrazine was used), and 1 ppb (0.001 µg/ml) for simazine, as well as the highest levels found in Illinois water supplies: 18 ppb (0.018 µg/ml) for atrazine, 12 ppb (0.012 µg/ml) for cyanazine, and 4 ppb (0.004 µg/ml) for simazine. All the treatments, including controls, were analyzed after 48 hr of incubation. The levels of the herbicides tested did not result in any measurable effect on cell growth. At the end of the incubation time, the cells in all flasks, controls and treatments, were approximately 80% confluent.

### Whole-Cell Analysis

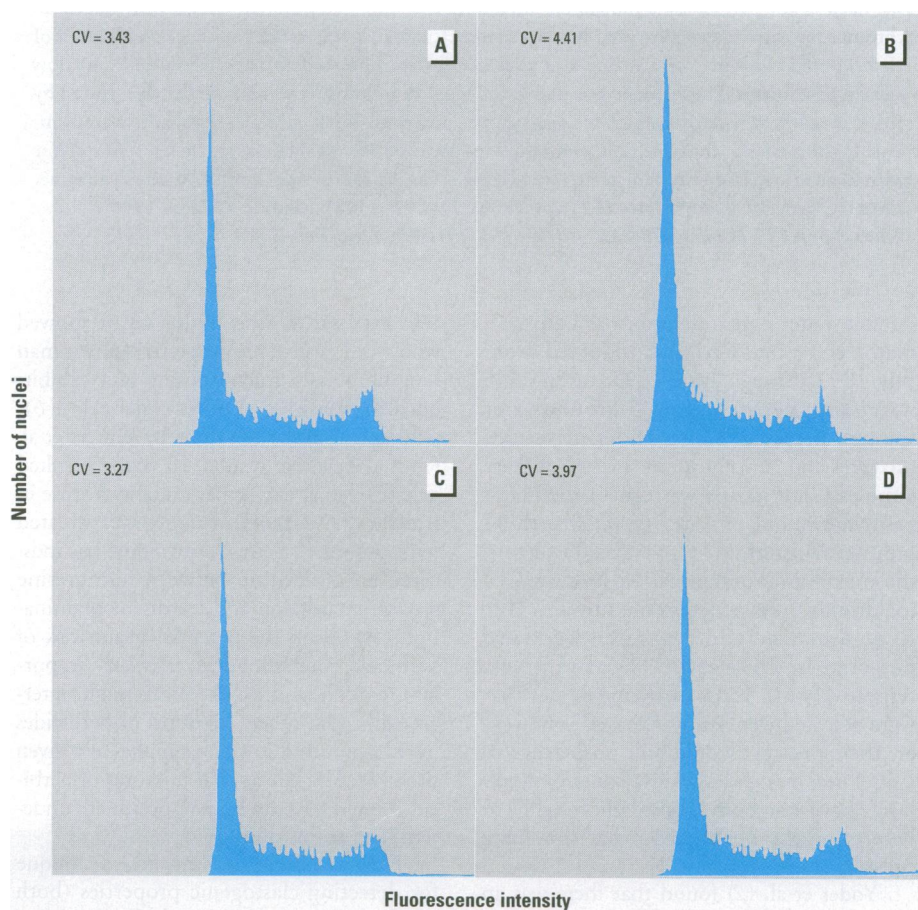
Nuclei were isolated from actively growing cell cultures by a propidium iodide (PI) hypotonic lysis method (21). The actively growing cultures were washed with sterile 1% PBS and then 1.5 ml of PI stain solution (0.05 mg/ml propidium iodide, 0.1% Triton X100, 0.1% sodium citrate, 7.0 units/ml RNase) was added to the cultures. The plates were refrigerated for 30–40 min, tilting every 3–5 min to release the nuclei from the bottom of the plate. The stain solution was then filtered through a 53-µm mesh filter and kept on ice until analysis. The nuclei were then analyzed with a Coulter EPICS 750 series flow cytometer-cell sorter (Coulter Electronics, Hialeah, FL). The excitation wavelength (488 nm) was provided by a 5-W argon ion laser. Epics-alignment

**Table 1.** Statistical analysis of whole-cell clastogenicity

Treatment	Concentration (µg/ml)	<i>n</i>	CV
EPA maximum contamination level			
Control	—	12	3.06
Atrazine	0.003	6	3.42***
Cyanazine	0.003	6	3.17
Simazine	0.001	6	3.26**
Atrazine/cyanazine	0.003/0.003	6	3.32**
Atrazine/simazine	0.003/0.001	6	3.34***
Simazine/cyanazine	0.001/0.003	6	3.19
Atrazine/simazine/cyanazine	0.003/0.001/0.003	6	3.29***
Highest level found in Illinois water supplies			
Atrazine	0.018	6	3.36***
Cyanazine	0.012	6	2.11
Simazine	0.004	6	3.35***
Atrazine/cyanazine	0.018/0.012	6	3.36***
Atrazine/simazine	0.018/0.004	6	3.37***
Simazine/cyanazine	0.004/0.012	6	3.21*
Atrazine/simazine/cyanazine	0.018/0.004/0.012	6	3.15

CV, coefficient of variation.

Significantly different from control at \**p* = 0.1, \*\**p* = 0.05, and \*\*\**p* = 0.01 levels.



**Figure 1.** Representative DNA histograms of (A) control nuclei, (B) nuclei exposed to 0.018 µg/ml atrazine, (C) nuclei exposed to 0.012 µg/ml cyanazine, and (D) nuclei exposed to 0.018 µg/ml atrazine and 0.004 µg/ml simazine.

Fluorospheres were used for optical alignment of the flow cytometer. The spheres were run immediately before data collection, once during data collection, and after data collection. Approximately 5,000 nuclei per sample were analyzed. The samples were run blind.

The histograms obtained in each analysis were analyzed manually, and the coefficient of variation (CV) of the G1 peaks was recorded. The borders surrounding the peaks were set manually by visually comparing the peaks, taking any abnormalities into



account. An analysis of variance (ANOVA) was performed on the CVs of the various treatments. A least significant difference (LSD) analysis was performed on the mean CVs of the various treatments to obtain the statistical results. Significance was designated at  $p = 0.10$ ,  $0.05$ , and  $0.01$ . Although it is customary to designate only  $p$  levels below  $0.05$ , in this instance the trends and the potential effects of any contamination on whole-cell clastogenicity warrant a more conservative approach before declaring that a contaminant has no effect.

### Flow Karyotype

The CHO cells were maintained as stated above. The chromosomes were isolated for analysis by the following procedure. Treatment cultures were prepared by putting a 1-ml cell suspension into petri plates containing the appropriate contaminants and 9 ml of complete media. The cells were then incubated for 48 hr. After the incubation period, the plates were checked microscopically for approximately 80% confluency. Media was then replaced with 10 ml complete media containing colcemid ( $0.1 \mu\text{g/ml}$ ) and the petri plates were incubated for 3–5 hr. Mitotic cells were dislodged by gentle shaking and were collected and centrifuged for 6 min at  $4^\circ\text{C}$ , 900 rpm. The pellet was resuspended in residual volume, and 0.5 ml staining solution I (75 mM KCL and  $50 \mu\text{g/ml}$  PI) was added. The tubes were then incubated for 10 min at  $37^\circ\text{C}$ , and 0.25 ml staining solution II (75 mM KCL,  $50 \mu\text{g/ml}$  PI, and 1% Triton X-100) was added. The tubes were then incubated at room temperature for 3 min. The samples were stored on ice until analysis. The separation of the chromosomes and breakage of the cells was achieved by pushing the sample through a 22-gauge needle. The procedure was adapted from Biradar and Rayburn (11). The chromosomes were then analyzed on a Coulter EPICS 750 series flow cytometer-cell sorter. The excitation wavelength (488 nm) was provided by a 5-W argon ion laser. Approximately 100,000 nuclei per sample were analyzed.

Flow karyotyping histograms were analyzed by taking the CV of the largest chromosome. The borders around the largest chromosome were set manually. An ANOVA was performed on the CVs of the various treatments. An LSD analysis was performed on the mean CVs of the various treatments to obtain the statistical results. Significance was designated at  $p = 0.10$ ,  $0.05$ , and  $0.01$ . As stated above, the potential effects of any contamination on chromosome damage warrant a more conservative approach before declaring a contaminant has no effect.

## Results

### Whole Cell

The cell cultures were incubated for 48 hr. The cells doubled every 12 hr, allowing for a maximum of four doublings. Table 1 and Figure 1 show the results of the whole-cell analysis. Cells exposed to atrazine, at both the MCL and at the highest level found, showed significantly higher CVs than the control ( $p = 0.01$ ; Fig. 1B). This agrees with the data previously presented by Biradar and Rayburn (5), who found that cells exposed to atrazine at the MCL had significantly higher CVs. They also ruled out the idea that the increase in CV could be due to the physical presence of atrazine in the nuclei (5).

The CVs from CHO cells treated with cyanazine, on the other hand, did not vary significantly from the control (Fig. 1C). Cells treated with simazine at the MCL had a moderately higher CV than the CVs of the control ( $p = 0.05$ ), but at the level of 4 ppb ( $0.004 \mu\text{g/ml}$ ), the cells showed a larger change in the CV of the G1 peak ( $p = 0.01$ ). This is in contrast to the results of Biradar and Rayburn (5), who found no effect after treatments with simazine for 48 hr.

When atrazine and cyanazine were mixed at the MCL level recommended for atrazine and at the highest levels found, the CVs of the treated cells showed significant variation from the controls ( $p = 0.01$ ). Atrazine mixed with simazine, at both levels tested, resulted in cells with an increase in the CV as well ( $p = 0.01$ ; Fig. 1D). The cyanazine and simazine mixture at the highest level reported in Illinois water supplies and mixtures of all three triazines at the MCL led to an increase in the CVs of the G1 peak of the CHO cells ( $p = 0.10$  and  $0.01$ , respectively). When simazine was mixed with cyanazine at the MCL and when all three herbicides were combined at the highest contamination level, no significant difference in the CVs of the G1 peak of the CHO cells was noted.

### Flow Karyotype

Table 2 and Figure 2 show the results from the flow karyotype of the CHO chromosomes. The CHO cells were incubated with the chemicals for 48 hr before analysis. Cells exposed to atrazine at both the MCL and at the highest level found in Illinois water supplies showed a significantly higher CV in the largest chromosome peak ( $p = 0.01$  and  $p = 0.10$ , respectively; Fig. 2C). This chromosome peak contained approximately 5,500 chromosomes. These data agree with those of Biradar and Rayburn (11).

The chromosomal CVs from the CHO cells treated with cyanazine had slight,

nonsignificant increases at the levels tested (Fig. 2D). The nonsignificance of the increase was due in part to the variability of induced chromosome damage. Although histograms from several replications demonstrated chromosome damage (Fig. 2F), other histograms showed no damaged chromosomes. Thus, the mean CV of the cyanazine treatment was below statistical significance. Chromosomes from cells treated with simazine at both levels tested also had slight, nonsignificant increases in CV. These results are in agreement with those of Biradar and Rayburn (5,11).

When atrazine was mixed with cyanazine, at both levels tested, a significant change in the CV of the largest chromosome peak was observed ( $p = 0.01$ ,  $p = 0.05$ ; Fig. 2E). Atrazine combined with simazine resulted in significant effects on the chromosomes at both levels tested ( $p = 0.05$ ). When simazine and cyanazine were combined, nonsignificant increases in the CVs were noted at both levels (Fig. 2B). When all three triazines were mixed, no significant increase in the CVs of the largest chromosome peak was observed.

## Discussion

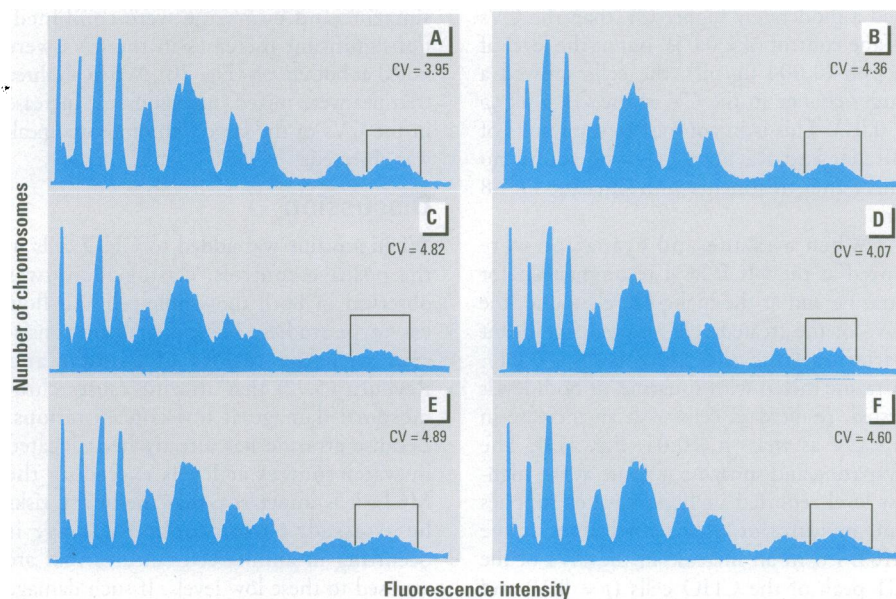
When atrazine was added to CHO cells in the positive controls, clastogenicity was observed in both the whole-cell and flow karyotype studies. These results are consistent with the results of Biradar and Rayburn (5,11) that atrazine causes chromosomal damage at low concentrations. Because atrazine has already been located in water sources at levels exceeding the MCL, it is imperative that the health risks be assessed. Chromosomal damage is occurring in animal cell cultures that are exposed to these low levels. If such damage is occurring in CHO cells, how might these levels affect human chromosomes? Do other triazines show similar effects? How do combinations of these chemicals react with chromosomes? The results of this study, as well as other studies, demonstrate that a potential human health risk may be associated with herbicide contamination, but it is beyond the scope of the present study to prove conclusively that such a health risk is present.

The results with both simazine and cyanazine indicate that compounds within the triazine class can react differently with respect to chromosome damage. Cells exposed to cyanazine alone did not reflect any statistically significant whole-cell clastogenicity nor damage to the flow karyotype. These findings may indicate that 1) the levels of cyanazine were not high enough to cause significant damage, 2) cyanazine does not have the potential to cause large amounts of

**Table 2.** Statistical analysis of chromosomal damage

Treatment	Concentration (µg/ml)	n	CV
EPA maximum contamination level			
Control	—	12	4.08
Atrazine	0.003	6	4.45***
Cyanazine	0.003	6	4.18
Simazine	0.001	6	4.22
Atrazine/cyanazine	0.003/0.003	6	4.45***
Altrazine/simazine	0.003/0.001	6	4.38**
Simazine/cyanazine	0.001/0.003	6	4.27
Atrazine/simazine/cyanazine	0.003/0.001/0.003	6	4.27
Highest level found in Illinois water supplies			
Atrazine	0.018	6	3.32*
Cyanazine	0.012	6	4.25
Simazine	0.004	6	4.17
Atrazine/cyanazine	0.018/0.012	6	4.38**
Altrazine/simazine	0.018/0.004	6	4.35**
Simazine/cyanazine	0.004/0.012	6	4.25
Atrazine/simazine/cyanazine	0.018/0.004/0.012	6	4.10

CV, coefficient of variation.

Significantly different from control at \* $p = 0.1$ , \*\* $p = 0.05$ , and \*\*\* $p = 0.01$  levels.**Figure 2.** Representative flow karyotyping histograms of (A) control chromosomes, (B) chromosomes exposed to 0.003 µg/ml cyanazine and 0.001 µg/ml simazine, (C) chromosomes exposed to 0.003 to 0.003 µg/ml atrazine, (D) chromosomes exposed to 0.003 µg/ml cyanazine, (E) chromosomes exposed to 0.003 µg/ml cyanazine and 0.003 µg/ml atrazine, and (F) chromosomes exposed to 0.012 µg/ml cyanazine. Brackets are around the largest chromosomes from which the CVs were taken.

chromosome damage, 3) any damage is restricted to a few chromosomes and therefore is only reflected in specific chromosome peaks in the flow histogram, or 4) cyanazine causes damage at other toxic end points. Due to the variability in the histograms of the cyanazine-treated cells, points 1 and 4 are the most probable explanations.

Unlike atrazine and cyanazine, simazine did not demonstrate a consistent chromosomal response. In previous studies, simazine was reported not to cause whole-cell clastogenicity at the MCL level (5,11). In this study, both levels of simazine tested gave positive results with respect to whole-cell

clastogenicity. No significant chromosome damage was observed in the flow karyotype. It appears that simazine, at the levels examined in this study, can cause whole-cell clastogenesis but not individual chromosome damage as measured by changes in the largest chromosome peak in the flow karyotype. Alternatively, any chromosome damage may be nonrandom, and thus the largest chromosome peak may not be affected. The concentrations of simazine used in this study may be at the threshold where chromosomal damage occurs. This would explain the apparent differences between the present studies and those of Biradar and

Rayburn (5,11). In attempting to determine if simazine causes more or less damage than atrazine, it is important to assess similar levels of the herbicides. The most reasonable comparison is between atrazine at 3 ppb (0.003 µg/ml) and simazine at 4 ppb (0.004 µg/ml). In both the whole-cell and flow karyotype studies, the CVs of the atrazine-treated cells were higher than the simazine-treated cells.

When atrazine is combined with simazine or cyanazine, there is significant clastogenic damage at the whole-cell level. However, the damage is apparently not increased over the amount of damage induced by atrazine alone: the amount of damage at the whole-cell level was equivalent to or less than the damage caused by atrazine alone. With respect to the flow karyotype data, the results were similar to those seen at the whole-cell level. The four tests in which the herbicides were combined indicate that no synergism occurs between the chemicals. The possibility of synergy between atrazine and the other triazine herbicides exists (see below) but is not readily observable at this level of flow analysis.

At the whole-cell level, the combination of simazine and cyanazine at MCL levels did not cause damage. However, when the concentration of the two chemicals were at the highest level found in Illinois water supplies, whole-cell damage occurred. When simazine and cyanazine are combined at both the MCL and the highest contamination level found in Illinois, there is little observed effect on the chromosomes of the CHO cells as observed in the flow karyotype. When the two chemicals were combined, there was little change in the CVs that were induced by the herbicides individually, indicating no synergism at the chromosome level at the concentrations tested.

For mixtures of the three herbicides, in both the whole-cell and flow karyotype studies, the CVs of the measured peaks decreased at both levels tested, indicating a decrease in clastogenic damage. We would have expected clastogenic damage to be present, if only due to the atrazine in the samples. The opposite appeared to be true. The CVs of the peaks examined were similar to the CVs of the controls in both the whole-cell and the flow karyotype studies. There appears to be an antagonistic effect on the damage usually caused by atrazine. An alternative hypothesis is that the combined levels of these herbicides result in selection for specific cell types that can grow in this acute environment. This would result in homogeneous cells with lower CVs. The tests would no longer be



the low-level chronic tests they were designed to be. Morphological observation of cells in the combined treatment support the acute test theory. The cells appear in distress compared with the control cells. This distress may be indicative of toxicological antagonism at an end point other than chromosomal damage. Two recent studies have observed a similar phenomenon in whole animal studies and reached similar conclusions (18,22). This hypothesis is further supported by the observation that when all three herbicides are combined at the lower concentrations, the CVs are higher than the CVs of cells exposed to the same trio of chemicals at a higher concentration (see Tables 1 and 2). Further studies are under way to determine if either of these two hypotheses is correct.

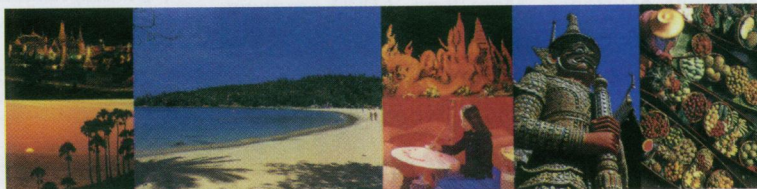
In conclusion, no synergistic effect with respect to clastogenic damage was observed when the triazine herbicides were tested in various combinations. With the potential of contamination with two or more triazine herbicides extremely high, it was imperative to test how these herbicides might interact to induce chromosome damage. Flow cytometry allows for a rapid, accurate assessment of the genome. For instance in the flow karyotype study, 33,000 chromosomes were examined per treatment. The time and effort to microscopically analyze these chromosomes would be prohibitive. It should be remembered that these tests do not reflect long-term exposure, nor do they suggest that synergy does not occur at other end points. In fact, data from this study suggest that a possible synergistic response at an alternative end point could be masking any chromosomal damage that

might be occurring. These results also do not pinpoint where the chromosomal aberrations occurred. Further studies are under way to more precisely define the whole-cell clastogenicity of short-term exposure and to study the long-term effect of these herbicides when they interact with one another.

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